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GROWTH OF PLANT CELL CULTURES

III. Growth Kinetics and Mass Culture

by

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## FOREWORD

This report represents the final phase of the work on culture of cells of higher plants undertaken at Natick under the Unconventional Food program in the Microbiology Division of the Food Laboratory. The objective of this study was to determine whether it would be feasible to use plant cell cultures as a source of human food. The objective of this phase was to utilize the cultures and media developed in the first two phases for large-scale growth and production of plant cell mass.

## Acknowledgement

Miss Karen Cohen and Mr. Richard Levy, summer student aids, did many of the cell counts for us. The inoculation device described on page 5 and illustrated in Fig. 2A was suggested by Mr. Isao Okada of this laboratory.

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### ABSTRACT

Suspension cultures of bean and lettuce cells have been maintained by serial transfer for over three years. Such cultures may show exponential growth, although growth rates are low with doubling times of three to four days. These suspension cultures have also been grown in commercial laboratory fermenters in batch and semi-continuous systems for periods of up to 61 days without contamination. Productivity in fermenters was up to 2.3 grams dry weight of cells per liter of culture per day. Cell yields are up to 40% based on the weight of sucrose in the medium.

## Introduction

The objective of this study was to determine the feasibility of growing plant cell cultures as a source of human food. Two previous reports (3, 4) have described the isolation of cultures, selection of media, and the nutrient requirements of plant cells growing in suspension. This report describes experiments with mass culture.

## Materials and Methods

The cultures and media and methods used in this work have been previously described (3, 4). All of the present studies were done using suspension cultures of Bean No. 8 and Lettuce No. 13 maintained in shake flasks by transfer (10% v/v) at approximately 3-week intervals using Murashige medium with 3.0% sucrose and 0.05 mg/L of dichlorophenoxyacetic acid (M) for bean, or 0.10 mg/L of naphthaleneacetic acid (NAA) for lettuce. In a few tests (as noted) Phytone at 1 g/L was added to M medium. Cultures were grown on rotary shakers at 26-28°C in low intensity white fluorescent light programmed 16 hours on, 8 hours off. The stock cultures were transferred over 55 times in a 3-year period. Under this routine maintenance, they sustained a productivity of 0.50 to 0.60 mg increase in dry weight per ml of culture per day. They also became stabilized as good suspension cultures growing as free cells and small clumps with little tendency to form large tissue masses.

All cultures and samples were routinely checked for sterility by streaking on nutrient agar and potato dextrose agar. Sterility checks were incubated for 7 to 10 days and then examined for growth. Contaminated cultures were discarded, and their data were not used. No antibiotics or antiseptics were added to the medium in any of our studies.

For growth studies, twenty flasks (50 ml per 250 ml Erlenmeyer) were inoculated from a single stock flask using a 5% v/v inoculum. Two flasks were harvested at once for initial values, and then two flasks at a time for subsequent values. Two 10-ml samples were removed from each flask for dry weight determinations. Cells were filtered on tared filter paper circles, washed with water, and dried at 70°C. The residues were pooled for microscopic study. The sample was diluted with water to about 1 to  $5 \times 10^3$  cells per ml. One-tenth ml of this dilution was mounted on a slide and all cells counted under the high dry objective. Although many cell clumps occur, with practice the number of cells in a clump can be estimated with fair accuracy. The major source of error is not in the count, but in taking a representative sample, and maintaining a uniform suspension during the dilution. Ten "typical" cells were measured with an ocular micrometer for average cell size. However, cell size and shape varied widely in all cultures. In other

experiments a pair of 1000 ml cultures was set up in 3 L Fernbach flasks, inoculated from a single 100 ml flask (50 ml inoculum = 5% v/v) and samples removed aseptically for dry weight determination and microscopic examination.

Reducing sugar was determined as glucose by a dinitrosalicylic acid method (6). For protein determinations, an aliquot of the culture was removed, the cells filtered off, washed with water, and homogenized in 0.1 N NaOH using a Virtis homogenizer. Protein in the homogenate was measured by the Folin method of Lowry et al. (1) using crystalline Bovine Plasma Albumin as a standard.

Fermenter studies were done using a New Brunswick Continuous Culture Apparatus (Fig. 1) with 5 to 15 liter Pyrex fermenter jars and stainless steel heads, impellers, baffles, cooling coils, and other fittings. Culture temperatures were maintained at 26° to 28°C by immersing the fermenter jar in a heated water bath at 28°C. Cold tap-water circulated through the cooling loop if the temperature in the sensing well rose above 27°C. Impeller speed was set at 120 RPM with a 3-inch diameter set of impeller blades low in the culture, and a second set of impeller blades just above the liquid level to aid in foam breaking. A few drops of Dow Corning Antifoam AF at 25 mg/ml was added manually as required. The air supply from a compressor was passed through a cotton filter (Fulflo Filter B3A), a packed alumina column of a trinity drier, a 0.22 micron Millipore filter, and finally through a 1 X 4 inch oven sterilized filter packed with a Seitz filter pad plus absorbent cotton, then injected to the culture through an orifice directly under the lower blades at about 1 to 1.5 liters per minute. A 0.30 inch 316 stainless steel wire coiled in the orifice was continued through the air inlet port and connected to a sleeve inside the air inlet hose. By manipulating the sleeve through the hose the coil could be moved back and forth to remove tissue masses that grew or were caught in the orifice, without opening the fermenter (Fig. 2B).

Contamination through the impeller shaft port was prevented by a loose fitting sleeve into which live steam was continuously passed (Fig. 2C). The exhaust air entered a 2 L Erlenmeyer flask trap, and then bubbled through one liter of 20% sulfuric acid in a second 2 L Erlenmeyer flask. Air inlet and exhaust lines on the nutrient reservoir and the harvest vessel were plugged by 1 X 4 inch oven sterilized filters packed with absorbent cotton.

For continuous operation (Fig. 1) the nutrient reservoir was a 15 L fermenter jar, stirred but not aerated or cooled, immersed in the same water bath as the 15 L culture vessel. A 20 L carboy in the refrigerator well, maintained at 5°C, served as the harvest vessel. The three vessels were connected by means of 5/8 inch gum rubber tubing inserted in the peristaltic pumps. The harvest pump was manually activated as desired. To prevent clogging and stagnation, the hose was reversed and back pumped at the beginning and end of each harvest to clear the line. A tap in the harvest line was used to collect samples. In the initial studies (constant volume) the

Fig. 1. Fermenter set up for growth of plant cell cultures

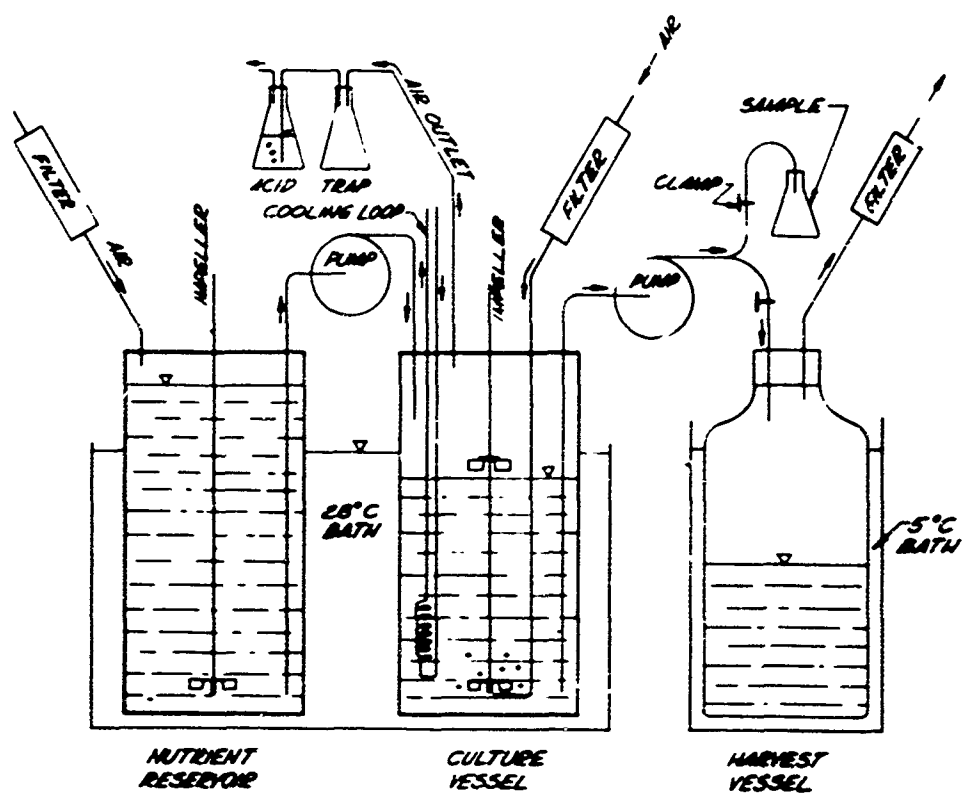
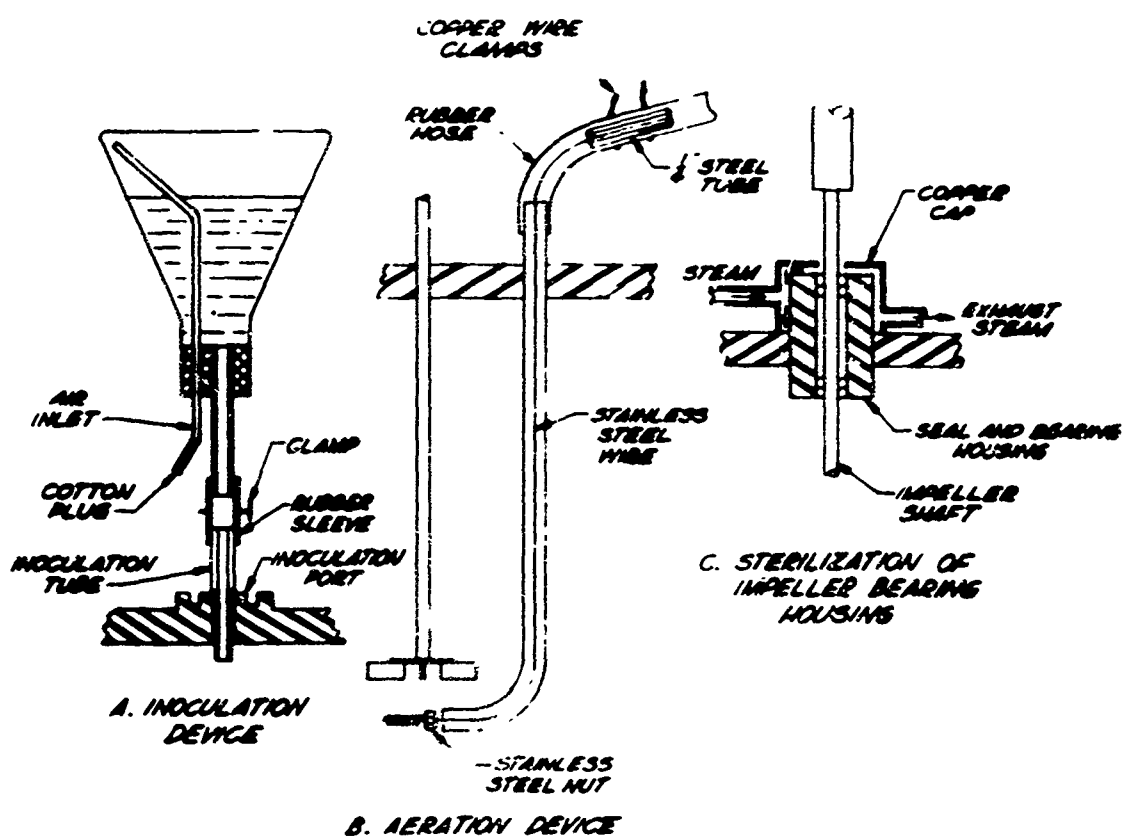


Fig. 2. Special devices used with fermenter set up for growth of plant cell cultures



nutrient pump was also operated manually to replace whatever volume of culture had been harvested. In later experiments (constant nutrient feed) nutrient solution was added at a steady rate of one liter or more per day through 1/4-inch gum rubber tubing using a New Brunswick AC-6 peristaltic pump activated every 2 minutes by a Cramer Timer for a time period set to give whatever daily volume was required. In these constant nutrient feed experiments the culture volume was allowed to increase for one to three days and then the harvest pump was run to reduce the culture to its original volume.

This set up was autoclaved intact and complete, except for the 3 air filters and the sulfuric acid in a New Brunswick autoclave. The nutrient reservoir and culture vessel were stirred during autoclaving and the autoclave temperature probe was inserted into the sensing well of the fermenter so that 30 minutes at 121°C was sufficient to sterilize volumes of up to 12 L of medium. Immediately after autoclaving, the 3 air filters were attached, and the sulfuric acid added in the exhaust vessel. As needed, new nutrient reservoirs or harvest vessels were autoclaved separately, the old one disconnected and the new one substituted. Open ends of hoses were wrapped with cotton and aluminum foil during autoclaving, and rinsed with ethanol as connections were made.

Inoculum for the fermenter was grown in or transferred to a 3 L Fernbach flask. The inoculum was transferred to the fermenter by a special device (Fig. 2A). This consisted of a 3 inch rubber stopper fitted with a 5/8 inch and a 1/4 inch stainless steel tube. The larger tube, for inoculum, was flush with the bottom of the stopper and extended 6 inches above it. It was connected by a short rubber sleeve to a second tube 7 inches long. The last 3 inches of the second tube was machined down to about 9/16 inches so that it fitted tightly into the inoculation port in the fermenter head. The smaller tube, for air, extended 7 inches (angled) above the stopper. After sterilization, this was fitted into the inoculum flask, replacing the cotton plug, the rubber sleeve was clamped off, and the Fernbach inverted over the fermenter. The wrappings were removed from the inoculum tube which was quickly inserted into the inoculation port, the clamp was opened, and the inoculum passed into the fermenter. A high level of success was achieved in inoculating and running fermenters for several weeks without contamination despite frequent samplings and changes of harvest vessels and nutrient reservoirs.

## Results

### Growth Studies in Flasks.

Exp. 1 - Growth of Bean Cells on Murashige Medium (Table 1, Fig. 3, 4).  
Dry weight increase lagged for 3 days and then increased exponentially until the 17th day, increasing 24 fold from 0.6 to 14.3 mg/ml with a doubling time

Table 1. Growth of Bean Cells on Murashige Medium

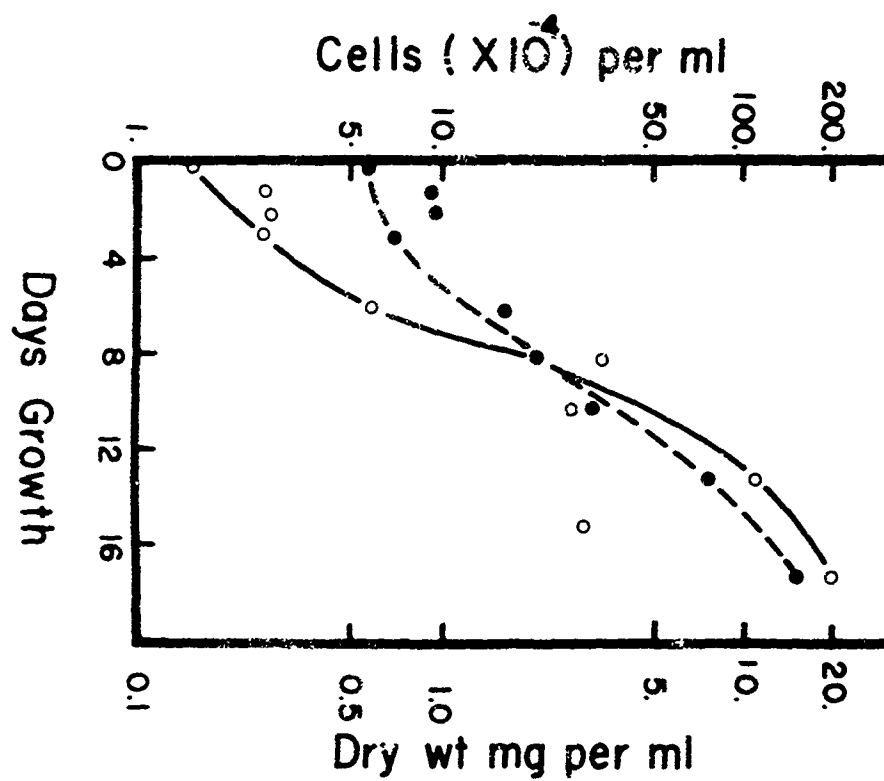
<u>Day</u>	<u>Dry wt</u> <u>mg/ml</u>	<u>Cells per ml</u>	<u>Cells</u> <u>per clump</u>	<u>Ave cell</u> <u>size</u> <u><math>\mu</math></u>	<u>mg/cell</u>
0	0.6	$1.4 \times 10^4$	3	80 X 88	39
1	0.9	2.6	6	60 X 81	35
2	0.9	2.6	6	74 X 89	36
3	0.7	2.5	5	75 X 100	27
6	1.6	5.9	4	60 X 78	27
8	2.0	13.4	10	63 X 85	15
10	3.1	12.6	18	56 X 71	24
13	7.6	18.0	23	53 X 65	7
15	11.3	29.5	21	63 X 84	39
17	14.3	195.0	10	57 X 72	7

Productivity = 0.80 mg/ml/day.

50 ml of culture per 250 ml Erlenmeyer, 5% v/v inoculum, 0.05 mg 2,4-D (2,4-dichlorophenoxyacetic acid) per L.

Fig. 3. Growth of bean cells on Murashige medium

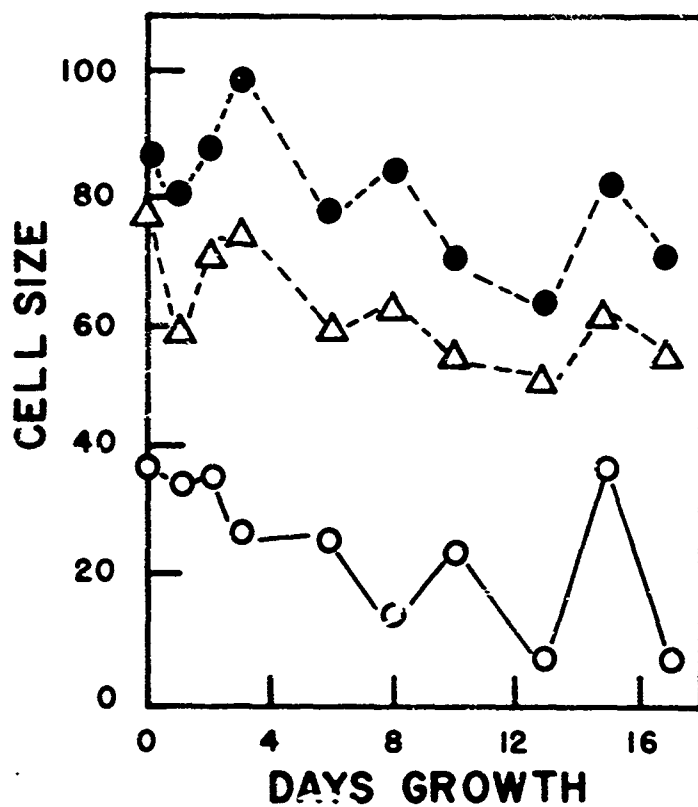
See Table 1 for details



● — dry weight  
○ — cell count

Fig. 4. Properties of bean cells on Murashige medium

See Table 1 for details



- average cell weight, micrograms
- - - ● average cell length, microns
- △- - - △ average cell width, microns

of about 3 days. Cell count increased 140 fold from  $1.4 \times 10^4$  to  $1.95 \times 10^6$ . Cells were initially disperse averaging 3 to 6 cells per clump through day 6, then increased to an average of 23 cells per clump by day 13. Cells averaged 5-10 micrograms in weight, 60-80 microns in width, and 70-100 microns in length.

Exp. 2 - Exponential Growth of Bean Cells on Murashige Medium with Phytone (Table 2, Fig. 5, 6). Growth was exponential for 14 days with a doubling time of about 3 days. Dry weight increased 15 fold from 0.6 to 9.3 mg/ml. Cell count increased 19 fold from  $2.8 \times 10^4$  to  $5.2 \times 10^5$  cells per ml. Cells occurred free and as moderate sized clusters. The cells averaged 10-30 micrograms in weight, 40-60 microns in width, and 60-90 microns in length.

Exp. 3 - Linear Growth of Bean Cells on Murashige Medium with Phytone (Table 3, Fig. 7). In this experiment, using a 1000 ml culture, despite a 68 fold increase in weight from 0.2 to 13.6 mg per ml, growth was linear with a steady increase of 0.75 mg per ml per day. This compares to productivity values of 0.80 for Exp. 1 and 0.66 for Exp. 2 where growth was mostly exponential. Thus, growth of bean cells may be exponential or linear without marked effect on the overall yield. Cell counts were not taken in this experiment.

Exp. 4 - Growth of Lettuce Cells on Murashige Medium with Phytone (Table 4, Fig. 8, 9). Cells grew exponentially for 12 days with a doubling time of about 4 days. Dry weight increased 12-fold from 0.7 to 8.4, cell count 22-fold from  $6.0 \times 10^4$  to  $1.29 \times 10^6$  cells per ml. Cells grew in small clumps and were smaller than bean cells, averaging 5-20 micrograms in weight, 40-60 microns in width, and 60-80 microns in length.

Exp. 5 - Growth of Lettuce on NAA Medium (Table 5, Fig. 10, 11). Cells grew exponentially for ten days with a doubling time of about 2.5 days. Dry weight increased 20-fold from 0.40 to 7.9 mg per ml. Cell count increased 50-fold from the 3rd to the 10th day from  $2 \times 10^5$  to  $1.05 \times 10^6$  cells per ml. Cells grew in clumps of 10 to 20, and averaged 4 to 8 micrograms in weight, 42-51 microns in width, and 55-62 microns in length.

#### Growth Studies in Fermenters.

Initial studies were done in batch usually using 3 liters of culture and inoculating with 100 to 1000 ml of a well-grown suspension culture of bean or lettuce cells. The most serious problems had to do with contamination at the time of inoculation, when taking samples, which entered via the air stream or around the impeller shaft. By trial and error the techniques were developed which solved the contamination problem without resorting to antibiotics. There were some problems with tissue growing in large masses, but this was largely eliminated by using the established suspension cultures which had less tendency to grow in such clumps. If the impellers were not used the cultures would not grow, but high impeller speeds damaged the large thin walled plant cells. Therefore, the lowest possible impeller setting, 120

Table 2. Exponential Growth of Bean Cells on Murashige Medium with Phytone

<u>Day</u>	<u>Dry wt</u> <u>mg/ml</u>	<u>Cells per ml</u>	<u>Cells</u> <u>per clump</u>	<u>Ave cell</u> <u>size</u> <u><math>\mu</math></u>	<u><math>\mu</math>g</u> <u>per cell</u>
0	0.6	$2.8 \times 10^4$	10	63 X 90	24
3	1.4	5.8	10	65 X 75	24
4	1.5	6.7	15	65 X 81	23
5	1.4	10.2	10	60 X 81	14
6	1.6	15.0	13	53 X 68	11
7	1.7	24.4	26	48 X 62	12
10	6.3	22.7	10	50 X 68	28
12	6.8	45.0	9	56 X 69	15
14	9.3	52.0	12	48 X 60	18
18	10.4	124.0	6	59 X 68	8
20	13.7	127.0	7	62 X 71	11

Productivity = 0.66 mg/ml/day.

50 ml culture per 250 ml Erlenmeyer, 5% v/v inoculum, 0.05 mg 2,4-D per L, 1.0 g Phytone/L.

Fig. 5. Exponential growth of bean cells on Murashige medium with Phytone

See Table 2 for details

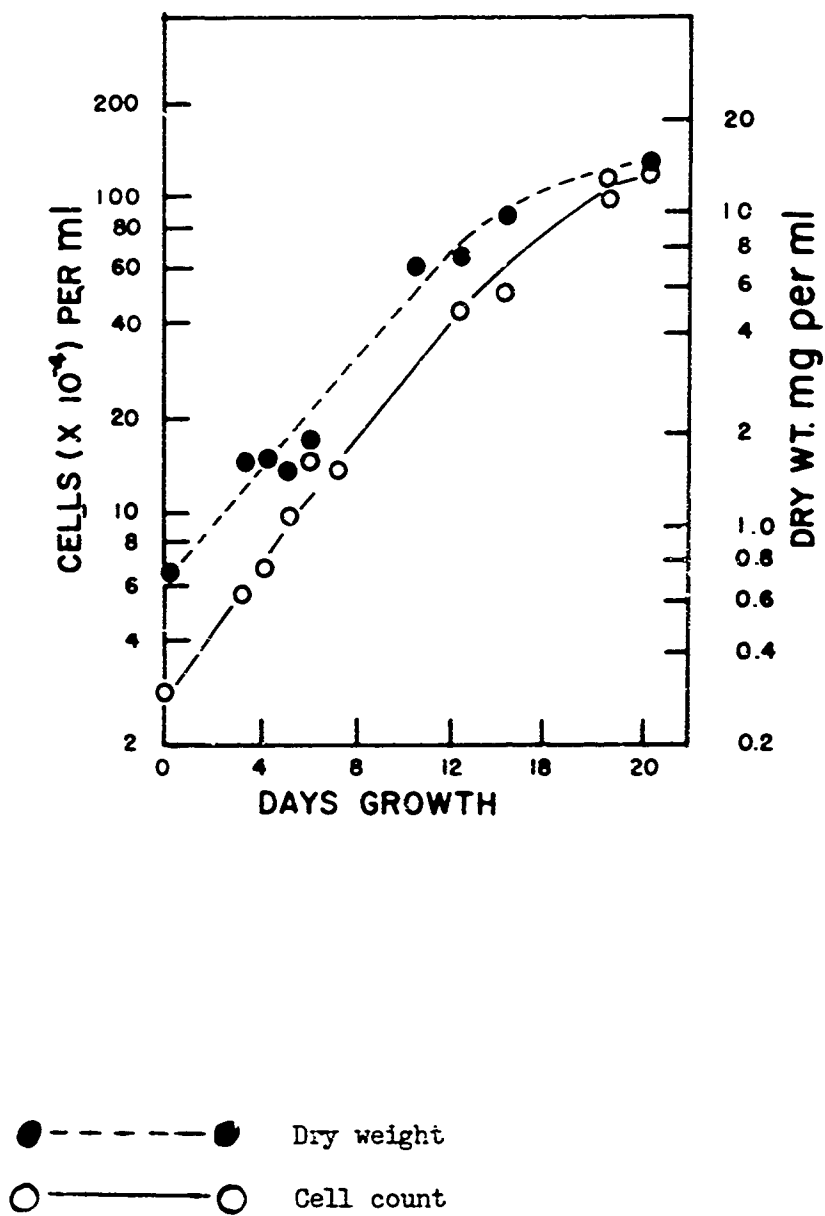
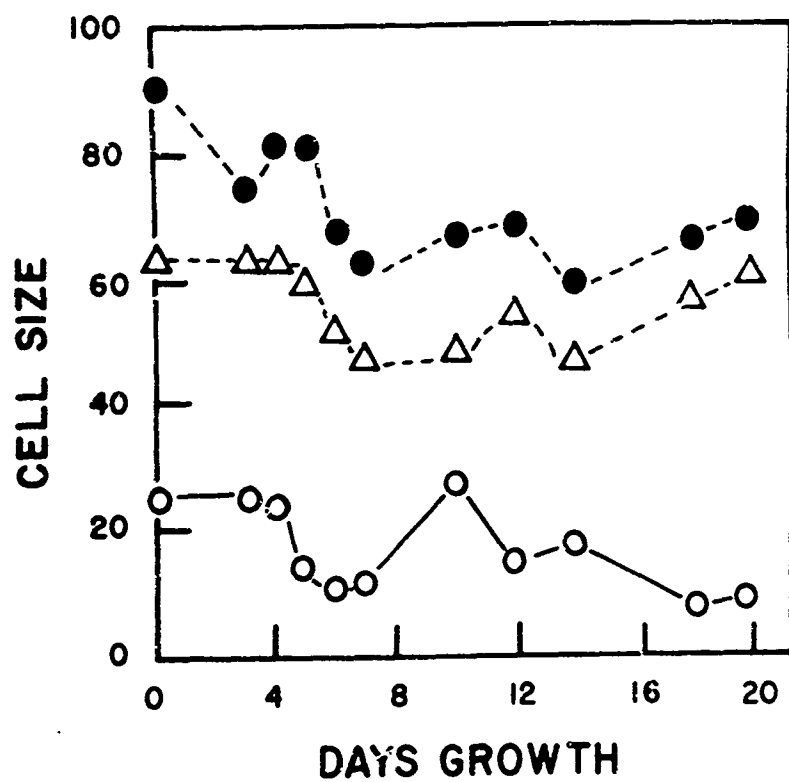


Fig. 6. Properties of bean cells on Murashige medium with Phytone

See Table 2 for details



- ——— ○ average cell weight, micrograms
- - - - - - ● average cell length, microns
- △ - - - - - △ average cell width, microns

Table 3. Linear Growth of Bean Cells on Murashige Medium with Phytone

<u>Day</u>	<u>Dry wt</u> <u>mg/ml</u>	<u>Generation</u> <u>time-days</u>	<u>Productivity</u> <u>mg/ml/day</u>
0	0.2	(from curve)	(from curve)
1	0.8	0.4	0.75
4	2.3	2.1	0.75
5	3.6	3.1	0.75
6	4.0	3.4	0.75
7	4.0	4.2	0.75
8	4.7	4.8	0.75
11	8.8	6.9	0.75
14	11.3	10.1	0.75
18	13.6	13.0	0.75

1000 ml culture in 3 L Fernbach, Inoculum 5% v/v, 0.05 mg 2,4-D per L,  
Phytone 1.0 g/L.

Fig. 7. Linear growth of bean cells on Murashige medium with Phytone

See Table 3 for details

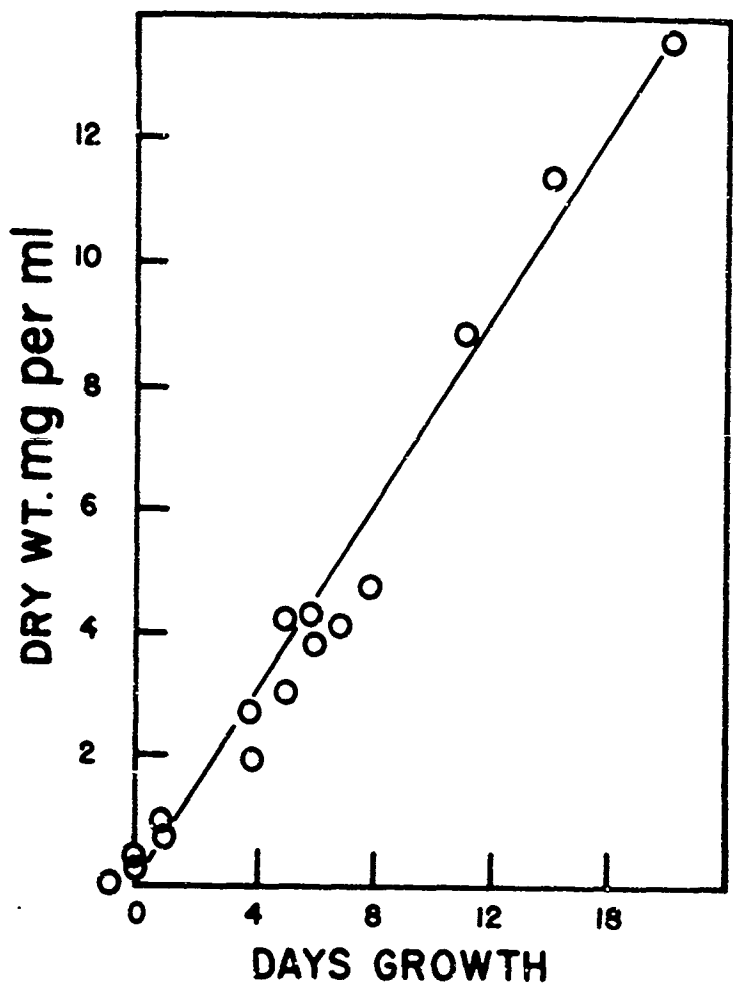


Table 4. Growth of Lettuce Cells on Murashige Medium with Phytone

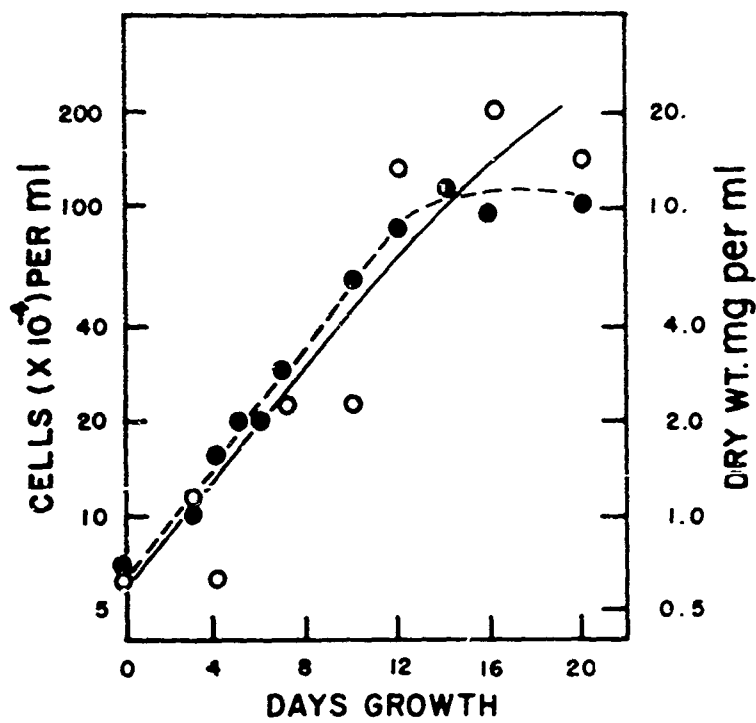
<u>Day</u>	<u>Dry wt</u> <u>mg/ml</u>	<u>Cells per ml</u>	<u>Cells</u> <u>per clump</u>	<u>Ave cell</u> <u>size</u> <u><math>\mu</math></u>	<u><math>\mu</math>g/cell</u>
0	0.7	$6.0 \times 10^4$	12	57 X 75	11
3	1.0	11.0	12	51 X 62	9
4	1.5	6.1	5	59 X 66	41
5	1.9	19.7	12	54 X 72	10
6	2.0	19.0	10	58 X 63	10
7	2.9	22.5	7	56 X 69	13
10	5.7	22.7	12	44 X 59	25
12	8.4	129.0	17	45 X 62	7
14	11.4	117.0	5	42 X 56	10
18	9.8	203.0	10	47 X 66	5
20	10.1	143.0	10	53 X 75	5

Productivity = 0.47 mg/ml per day.

50 ml culture per 250 ml Erlenmeyer, inoculum 5% v/v, 2,4-D 0.05 mg/L, Phytone 1.0 g/L.

Fig. 8. Growth of lettuce cells on Murashige medium with Phytone

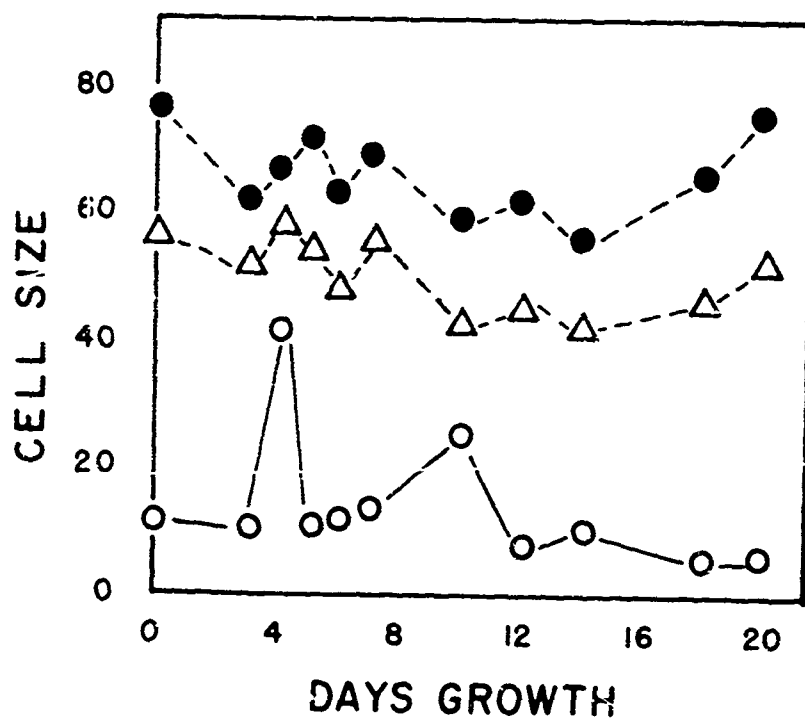
See Table 4 for details



● ——— ● dry weight  
○ ——— ○ cell count

Fig. 9. Properties of lettuce cells on Murashige medium with Phytone

See Table 4 for details



- ————— ○ average cell weight, micrograms  
 ● ————— ● average cell length, microns  
 △ ————— △ average cell width, microns

Table 5. Growth of Lettuce Cells on NAA Medium

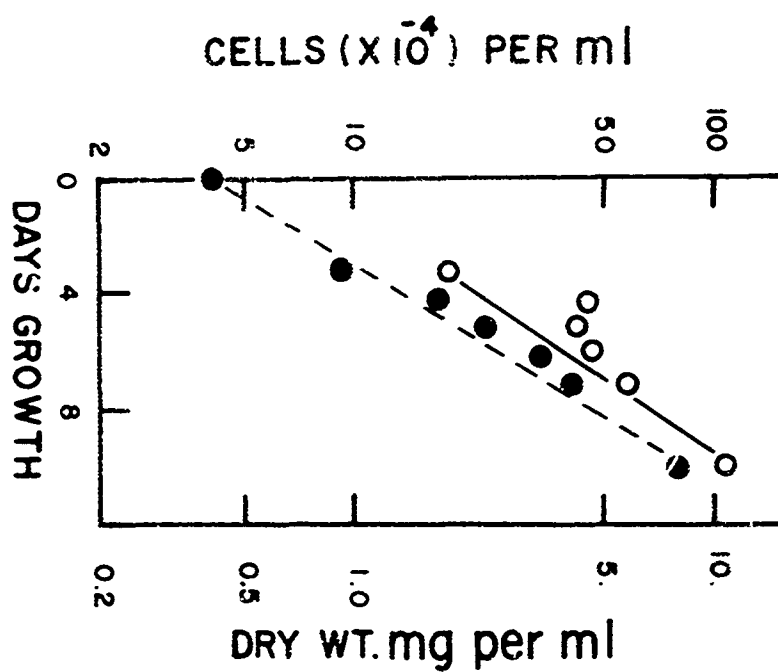
<u>Day</u>	<u>Dry wt</u> <u>mg/ml</u>	<u>Cells per ml</u>	<u>Cells</u> <u>per clump</u>	<u>Ave cell</u> <u>size</u> <u><math>\mu</math></u>	<u><math>\mu</math>g/cell</u>
0	0.4	-----	--	---	---
3	0.9	$19.7 \times 10^4$	13	$46 \times 55$	5.0
4	1.5	35.4	14	$45 \times 57$	3.9
5	1.8	23.4	10	$51 \times 62$	5.6
6	2.4	65.4	20	$48 \times 58$	6.7
7	2.1	37.4	11	$47 \times 55$	7.0
10	7.9	105.0	10	$42 \times 56$	7.5

Productivity = 0.75 mg/ml per day.

1000 ml culture per 3 L Fernbach, Inoculum 5% v/v. NAA (naphthaleneacetic acid) 0.10 mg/L.

Fig. 10. Growth of lettuce cells on NAA medium

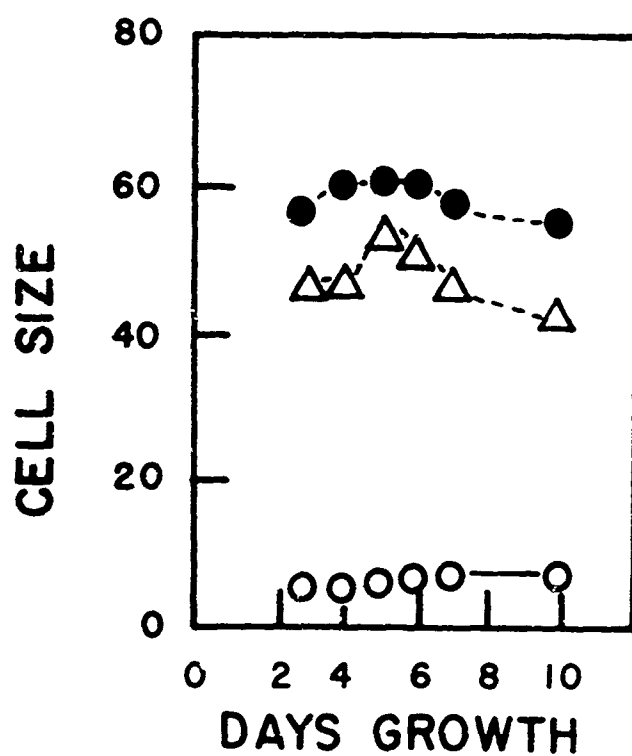
See Table 5 for details



● ——— ● dry weight  
○ ——— ○ cell count

Fig. 11. Properties of lettuce cells on NAA medium

See Table 5 for details



- ——— ○ average cell weight, micrograms  
● - - - - - ● average cell length, microns  
△ - - - - - △ average cell width, microns

revolutions per minute, was used. Because of the low impeller speed, and the low aeration rates, foam was not usually a serious problem. Occasionally, Dow Corning AF silicone antifoam was added at less than 10 mg/L. Tests with bean and lettuce cultures in shake flasks with this antifoam showed no increase or decrease in growth, nor any effect on cell size or clumping up to 35 mg/L, the highest level tested. Another problem was growth of cells in foam and on the walls of the vessel above the liquid level. Such cultures tended to have almost no growth in the liquid. The growth in the foam became dry and brown. The only solution for this problem was to disconnect the fermenter and shake it up manually to wash the cells back into the liquid. In successful batch cultures up to 6 g dry weight of cells per L were produced in 2 or 3 weeks. Because of the initial lag in growth, and because most of the problems were associated with getting cultures started, it was decided to use semicontinuous cultures where part of the culture would be periodically harvested and replaced with fresh nutrient. Although bean cells grew well in batch cultures, they were less successful in the semicontinuous cultures because their firm clumps tended to clog the harvest line. Lettuce cells which grow in smaller, more friable clumps did well under continuous culture. Three such experiments with lettuce are described.

Exp. 1 - Growth of Lettuce on NAA Medium in a Fermenter with Intermittent Harvest and Addition of Nutrient (Table 6). Three liters of NAA medium in a 15 L culture vessel was inoculated with 1 L of a well-grown lettuce culture. The inoculum totaled 10 g dry weight. Ten L of the same medium was used in the nutrient reservoir. At day 1 1.5 L of the culture was pumped into the harvest vessel, and 1.5 L of fresh nutrient was pumped into the culture vessel. This was repeated every 2 or 3 days as indicated in Table 6. The culture volume was maintained at 4 L for 26 days, and then was increased to 6.5 to 7.5 liters by pumping extra nutrient. New reservoirs were substituted at day 14 (10 L), day 23 (10 L), and day 33 (12 L). The harvest vessel was changed at day 23. Samples were taken regularly and pH, dry weight, and reducing sugar in the medium determined. The last was a measure of sugar consumed. Since lettuce cells secrete invertase (2) the sucrose in the medium is rapidly inverted. This culture grew well and remained uncontaminated for 41 days. On that day the culture was given a vigorous shake to wash down cells growing above the liquid level, and a new reservoir harvest vessel were attached. At the next sampling (Day 43) the culture was contaminated and was discontinued. In 41 days 40 L of culture was harvested containing 478 g dry weight of cells, an average of 12 g per liter. The average volume of the culture was 4.9 L, the average dilution rate was 0.20 per day, and the overall productivity 2.3 grams per liter of culture per day. The yield was 39% based on a net production of 468 g of cells from 1200 g of sucrose (40 L  $\times$  30 g/L). Growth lagged for the first 7 days and then increased. A steady state was not reached. Cell concentration in the harvest from day 12 to day 41 ranged from 7 to 19 mg per ml. Clogging of the harvest line or growth above the liquid level resulted in low cell concentrations in the harvest; after shaking the culture, cell

Table 6. Growth of Lettuce in a Fermenter with Intermittent Harvest and Addition of Nutrient - Experiment 1 - NAA 0.1 mg/L, Sucrose 3%, T 27°C, Air 1.5 L/Min, Impeller 120 RPM

Day	Culture Volume* liters	Harvest liters	pH	Reducing Sugar mg/ml	Dry Weight mg/ml	Harvest grams
0	4.0	---	5.0	---	2.5	-----
1	4.0	1.5	5.6	17.6	2.5	3.75
2	4.0	1.5	4.8	19.6	2.3	3.45
5	4.0	1.5	5.0	13.2	1.2	1.80
7	4.0	1.5	4.6	24.0	1.9	2.85
12	4.0	1.5	4.8	4.3	16.2	24.30
14	4.0	2.5	4.7	2.6	10.3	25.75
16	4.0	2.5	4.8	1.1	12.5	31.25
19	4.0	2.5	5.0	---	11.9	29.75
21	4.0	2.5	5.5	--	13.4	33.50
23	4.0	2.5	5.8	8.0	6.7	16.75
26	4.0	2.5	5.9	12.5	11.0	27.50
29	7.5	2.5	5.9	3.1	14.6	36.50
33	6.5	2.5	5.9	11.0	11.0	27.50
36	7.0	2.5	5.9	9.7	13.1	32.75
40	7.5	2.5	6.1	12.5	16.3	40.75
41	---	7.5	5.9	2.7	18.7	140.25

\* Constant volume, recorded after harvest and addition of nutrient.

$$\text{Productivity} = \frac{478.4 - 10 \text{ grams}}{41 \text{ days} \times 4.9 \text{ L}} = 2.3 \text{ g/L/day.}$$

concentration in the harvest was high. However, not all of the variation was an artifact as shown by fluctuations in pH and residual sugar.

The harvested lettuce cells were an attractive light green color and had a pleasant fruity flavor. They were filtered off from the medium, using 2 or 3 thicknesses of cheese cloth, washed with water, freeze dried, and canned under nitrogen. These cells and cells harvested from other successful fermenters have been saved for use in feeding tests. Casual observations have indicated that white mice readily eat fresh or freeze dried plant cells without any apparent ill effect.

Exp. 2 - Growth of Lettuce on MAA Medium with 3% Sucrose in a Fermenter with Continuous Nutrient Feed and Intermittent Harvest (Table 7). Three L of MAA medium in a 15 L culture vessel was inoculated with 1 L of a young lettuce culture. The inoculum totaled 4 g dry weight. Twelve L of the same medium was used in the nutrient reservoir. The culture was allowed to grow as a batch for 5 days and then nutrient was fed to the culture on a continuous basis at about 1 L per day. The nutrient feed was discontinued from day 9 to day 12 and then continued at 1 L per day until day 37 at which time it was increased to 1.5 L per day. In this experiment the culture volume was constantly changing. Initially 4 L, it had increased to 8 L by day 12, then 2 L was harvested reducing the volume to 6 L. By day 14 the volume had increased again to 9 L and 3 L were harvested reducing the volume again to 6 L and so on. However, because of the constant nutrient feed, changes in the medium in which the culture was growing were minimized, although the dilution rate was greater when culture volume was low. In experiment 1 the intermittent addition of fresh nutrient resulted in sudden marked changes in the composition of the culture medium.

New 12 L reservoirs were substituted on day 19, day 28, day 40, and day 47. Although the culture was growing well, considerable residual sugar remained in the medium. On day 54 a new reservoir was substituted with sucrose concentration reduced to 2%. The harvest vessel was changed on day 26, day 42, and day 54. Samples were taken regularly and pH, dry weight, and reducing sugar determined. The culture grew well and remained uncontaminated for 61 days. On day 61 a new reservoir was attached. On day 63 the culture was contaminated and was discontinued. In 61 days 75 L of culture were harvested containing 706 g dry weight of cells, an average of 9.5 g per Liter. The average volume of the culture was 7.1 L, the average dilution rate 0.15 per day, and overall productivity 1.6 grams per liter of culture per day. The yield was 34% based on a net production of 702 g of cells from 2100 g of sucrose (62 L X 30 g/L and 12 L X 20 g/L). This culture showed a long lag, perhaps because a young, rather light inoculum was used and a good cell concentration was not attained until day 16. After that, growth was good with cell concentrations in the harvest ranging from 6 to 18 mg/ml.

Table 7. Growth of Lettuce in a Fermenter with Continuous Nutrient Feed and Intermittent Harvest. Experiment 2. NAA 0.1 mg/L, Sucrose 3%, T 27°C, Air 1.5 L/min, Impeller 120 RPM

<u>Day</u>	<u>Culture volume* liters</u>	<u>Average volume liters</u>	<u>Feed rate L/day</u>	<u>Harvest liters</u>	<u>pH</u>	<u>Reducing sugar mg/ml</u>	<u>Dry weight mg/ml</u>	<u>Harvest grams</u>
0	4.0	---	0	0	5.0	----	1.0	0
2	4.0	4.0	0	0	6.0	18.0	0.9	0
5	4.0	4.0	1.0	0	5.6	24.8	0.5	0
7	6.0	5.0	1.0	0	5.3	28.0	1.1	0
9	8.0	7.0	0	0	4.8	----	0.8	0
12	8.0	8.0	1.0	2.0	5.0	9.0	2.4	4.8
14	9.0	7.5	1.0	3.0	5.2	6.0	4.1	12.3
16	8.0	7.0	1.0	2.0	5.3	8.4	7.3	11.6
19	9.0	7.5	1.0	3.0	5.0	9.8	11.4	34.2
21	8.0	7.0	1.0	2.0	4.8	11.0	11.3	22.6
23	8.0	7.0	1.0	2.0	4.2	12.8	17.8	35.6
26	9.0	7.5	1.0	3.0	4.2	10.0	9.6	28.8
28	8.0	7.0	1.0	2.0	4.8	9.7	9.0	18.0
30	8.0	7.0	1.0	2.0	4.2	10.0	8.9	17.8
33	10.0	8.0	1.0	4.0	5.0	9.3	12.9	51.6
35	9.0	7.5	1.0	3.0	4.8	9.3	10.2	30.6
37	8.5	7.3	1.0	3.0	4.8	9.3	11.7	35.1
40	8.0	6.8	1.5	2.0	4.2	9.2	13.4	26.8
42	9.0	7.5	1.5	3.0	5.1	13.8	9.1	27.3
44	9.0	7.5	1.5	4.0	4.4	16.6	8.2	37.8
47	10.0	7.5	1.5	4.0	4.8	21.0	7.4	29.6

Table 7. Growth of Lettuce in a Fermenter with Continuous Nutrient Feed and Intermittent Harvest. Experiment 2. NAA 0.1 mg/L, Sucrose 3%, T 27°C, Air 1.5 L/min, Impeller 120 RPM. (Cont'd)

Day	Culture volume* liters	Average volume liters	Feed rate L/day	Harvest liters	pH	Reducing sugar mg/ml	Dry weight mg/ml	Harvest grams
49	9.0	7.5	1.5	3.0	5.4	16.9	7.4	22.2
51	9.0	7.5	1.5	3.0	5.3	10.4	9.0	27.0
54	11.0	8.5	1.5	5.0	5.0	11.4	8.6	43.0
Reduce Sucrose concentration in Reservoir to 2.0%								
56	9.0	7.0	1.5	3.0	5.2	13.0	5.5	16.5
58	11.0	8.5	1.5	6.0	4.9	8.3	11.1	66.6
61	10.5	7.75	---	10.5	4.7	7.8	10.3	108.2

\* Before harvest.

$$\text{Productivity} = \frac{706 - 4 \text{ g}}{61 \text{ days} \times 7 \text{ l L}} = 1.6 \text{ g/L/day}$$

Exp. 3 - Growth of Lettuce on NAA Medium with 2% Sucrose in a Fermenter with Continuous Nutrient Feed and Intermittent Harvest (Table 8). Three L of NAA medium with 2% sucrose in a 15 L culture vessel was inoculated with 1 L of a well-grown lettuce culture. The inoculum totaled 9.7 g dry weight. The culture was allowed to grow for 5 days and then nutrient was fed into the culture on a continuous basis at about 1 L per day. The first harvest was taken on day 7 and continued on an intermittent basis as in experiment 2. New 12 L reservoirs were substituted on day 16, day 26, day 33, and day 42. The harvest vessel was changed on day 26 and day 40. This culture grew well for 44 days, but was discontinued on day 46 because of contamination. Samples were taken regularly as before, and pH, dry weight, reducing sugar, cell number, and protein concentration determined.

In 44 days 69 L of culture was harvested containing 560 g of cells, an average of 8.1 g per liter. The average volume of the culture was 6.1 L, the average dilution rate 0.26 per day, and the overall productivity 2.0 g/L of culture per day. The yield was 40% based on a net production of 550 g of cells from 1380 grams of sucrose (69 L X 20 g/L).

This culture showed a good cell concentration from day 7 on ranging from 5-12 mg/ml. Sugar was well utilized with residual sugar in the medium ranging from 0.03 to 3.9 mg/ml. Cell counts fluctuated rather widely from day 8 to day 28 but then stabilized at about  $22 \times 10^4$ /ml. Protein concentration in the harvested cells ranged from 8 to 19%.

The results of the three experiments are summarized in Table 9.

### Discussion

Plant cells that have been maintained as suspension cultures can grow exponentially, probably because in such suspension cultures the majority of cells remain capable of cell division. Growth rates remain low, and productivity is no greater than that obtained with cells showing Zero order increase. Since all nutrients are present in excess (4) and growth conditions appear favorable, the question arises as to what is limiting growth. One possibility is that plant cells are inherently incapable of more rapid growth. Another is that the limiting factor is the rate of synthesis of some compound or compounds by the cells themselves. In objection to this view is the failure to get any marked increase in growth rates by adding known growth factors or complex additives to the medium (3, 4). Another possibility is that the rate of diffusion of nutrients into the large plant cells is a limiting factor. If so, reduction of average cell size or clump size should lead to increases in growth rates.

Table 8. Growth of Lettuce in a Fermenter with Continuous Nutrient Feed and Intermittent Harvest. Experiment 3. NAA 0.10 mg/L, 2.0% Sucrose, T 27°C, Air 1.5 L/min, Impeller 120 RPM

Day	Culture volume* liters	Average volume liters	Feed rate L/day	Harvest liters	pH	Reducing sugar mg/ml	Dry weight mg/ml	Harvest grams	Protein mg/gram	Cells/ml X 10 <sup>-4</sup>
0	4.0	---	0	0	---	---	2.4	0	---	---
5	4.0	4.0	1.0	0	---	---	---	0	---	---
7	5.0	4.5	1.0	1.0	5.5	0.10	11.6	11.6	---	---
8	---	---	1.0	0	---	---	---	0	---	26.0
9	6.0	5.0	1.0	1.0	5.3	0.03	12.2	12.2	---	48.5
12	8.0	6.5	2.0	2.0	5.4	0.06	11.6	23.2	---	26.9
14	9.0	7.5	2.0	4.0	5.4	0.07	11.4	45.6	---	42.4
16	9.0	7.0	1.0	4.0	4.5	1.3	11.8	47.2	---	37.4
19	8.0	6.5	1.5	3.0	5.3	0.10	10.0	30.0	194	66.2
21	7.5	6.3	1.5	3.0	5.4	0.09	8.2	24.6	123	32.5
23	7.5	6.0	1.5	3.0	4.7	0.69	7.3	21.9	96	46.1
26	9.0	6.8	1.5	5.0	5.4	0.05	11.5	57.5	136	38.7
28	7.0	6.5	1.5	3.0	5.2	0.83	5.2	15.6	---	16.6
30	10.0	7.0	1.5	6.0	5.5	2.5	8.5	51.0	93	21.4
33	11.0	7.5	2.0	7.0	4.5	2.9	10.5	73.5	122	23.0
35	7.0	6.5	1.5	3.0	5.2	2.6	8.4	25.2	101	20.7
37	6.0	5.0	1.5	2.0	4.9	1.6	10.5	21.0	109	23.3
40	9.0	6.5	1.5	5.0	5.3	0.93	7.2	36.0	95	22.6
42	8.0	6.0	1.0	4.0	4.8	1.6	4.7	18.8	81	21.6
44	9.0	6.5	1.0	9.0	5.5	3.9	5.0	45.0	94	21.1

\* Before harvest.

$$\text{Productivity} = \frac{559.9 - 9.7 \text{ grams}}{44 \text{ days} \times 6.1 \text{ L}} = 2.0 \text{ g/L/day}$$

Table 9. Summary of Fermentation Experiments with Lettuce on MAA Medium

<u>Experiment Number</u>	<u>1</u>	<u>2</u>	<u>3</u>
Culture number	13	13	13
Sucrose Concentration - %	3.0	3.0 → 2.0	2.0
Inoculum - grams	10.0	4.0	2.7
Days operated	41	61	44
Average culture volume - liters	4.9	7.1	6.1
Ave. feed rate - liters/day	0.97	1.04	1.57
Harvest total - liters	40.0	74.5	62.0
Ave. concentration - grams/liter	12.0	9.5	8.1
Dilution rate per day (1)	0.20	0.15	0.26
Yield - grams/100 g sucrose (2)	39	34	40
Productivity - G per L per day (3)	2.3	1.6	2.1

(1)  $\frac{\text{Feed Rate}}{\text{Ave. volume}}$

(2)  $\frac{(\text{Total Harvest} - \text{grams}) - (\text{Inoculum})}{(\text{Total liters}) \times (\text{g sucrose/L})} \times 100$

(3)  $\frac{(\text{Total Harvest} - \text{grams}) - (\text{Inoculum})}{(\text{No. days}) \times (\text{Ave. culture volume})}$

In our first report (3) the various systems reported in the literature for producing large quantities of plant cells in liquid suspension culture were reviewed. A recent report (6) described a Phytostat designed for continuous culture and automatic sampling. In the present study there has been good success with semi-continuous culture of lettuce cells in a commercially available fermenter, modified to adapt it to plant cell culture. While a steady state has not been attained there has been a fairly steady rate of production of health uncontaminated cells for periods of up to 61 days. Productivity is higher than achieved in shake flasks. The system is ideal for producing large quantities of plant cells with a minimum of manipulation. Continuous nutrient feed has been used. Continuous harvest has not been attempted. Because of the thick suspension of large plant cells and clumps, the harvest line must be at least 0.5 inches in diameter. At the present slow feed rates, it would be very difficult to maintain a constant suspension in such a harvest line. In a very large set-up a continuous harvest would be possible.

In the present study, productivities have been maintained of 1.5, 2.0, and 2.3 grams per liter of culture per day in three experiments totaling 146 days. In a 10 liter fermenter it would take about 50 days to produce a kilogram of dry cells at these rates. Material costs would be low, about 2-1/2 kg of sucrose, plus nutrient salts, and very small quantities of inexpensive growth factors would be required. However, the costs of operation may be prohibitive at this stage of research. The possibility of using plant cells grown in a fermenter for food is small, except under circumstances where cost does not matter.

Growth of plant cells in a fermenter is however, an excellent means of accumulating cells for metabolic studies or for producing high cost products such as pharmaceuticals or enzymes. This set-up also provides an excellent possibility for studying effects of medium and other factors on growth rates and composition of plant cells. In time such studies may lead to increased rates of growth, and more economical production of plant cells.

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<p>Suspension cultures of bean and lettuce cells have been maintained by serial transfer for over three years. Such cultures may show exponential growth, although growth rates are low with doubling times of three to four days. These suspension cultures have also been grown in commercial laboratory fermenters in batch and semi-continuous systems for period of up to 61 days without contamination. Productivity in fermenters was up to 2.3 grams dry weight of cells per liter of culture per day. Cell yields are up to 40% based in the weight of sucrose in the medium.</p>		

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Cultures (Biology)	9		9			
Plant cells	9		9			
Beans	9		9			
Lettuce	9		9			
Culture media	9		9			
Flasks	9		9			
Fermenters	9		9			
Food	9		9			
Constraining	9		9			
Increasing	9		9			
Biological productivity	9		9			

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